MORPHOLOGIC VARIATION IN PNEUMOCOCCUS

I. An Analysis of the Bases for Morphologic Variation in Pneumococcus and Description of a Hitherto Undefined Morphologic Variant*

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Plates 4 and 5

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Since Dawson's description of a rough variant of pneumococcus (1), little has been written concerning the bases for morphologic variation in this species. Recent studies of the effect of transformation reactions upon the morphology of pneumococcus (2–4), however, have created renewed interest in this subject and have made desirable also reconsideration of the problems of colonial variation in bacteria.

It has been customary, in the past, to describe structural variation in the cells of a number of bacterial species in terms of its effect upon the appearance of colonies of such bacteria on the surface of solid media. The descriptive terms employed most widely, though not universally, to designate the several colonial forms have been mucoid (M), smooth (S), and rough (R). Although these terms have been helpful in the past in indicating certain patterns of bacterial variation, significant objections to their continued use have arisen. To begin, different terms have been applied to analogous variants of different bacterial species (1). Second, the colonial nomenclature of mucoid, smooth, and rough has come to imply, in certain instances, fixed relationships between independently variable bacterial characters, and for this reason, as pointed out by Topley and Wilson (5), much confusion has arisen, especially over the use of the terms smooth and rough. In addition, difficulty has been experienced in classifying forms which manifest simultaneously properties of both the rough and mucoid states.

Further problems have been created by the recognition of bacterial variants intermediate between mucoid and smooth and between smooth and rough. The production of surface polysaccharide by a bacterial cell may be subject to genetically controlled, quantitative variation and cells producing large amounts of polysaccharide may form typically mucoid (M) colonies whereas those producing minimal amounts of surface carbohydrate may give rise to colonies indistinguishable in the gross

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from the smooth (S) colonies of cells forming no polysaccharide (6). Cells forming even minimal quantities of polysaccharide, however, are better classified from a genetic and immunochemical point of view with other cells manifesting this activity than with those which do not. Difficulty concerning the term mucoid results from its application to bacterial surface carbohydrate whether recognizable as a capsule or distributed diffusely throughout the area of bacterial growth. Because certain bacteria, such as *Streptococcus salivarius*, may exist in both capsulated and non-capsulated forms and because both these variants may produce a mucoid, diffusely distributed polysaccharide differing from the capsular carbohydrate (7), designation of a mucoid colonial form becomes ambiguous when it might be used to describe more than one bacterial variant.

Distinction between smooth and rough variants presents problems analogous to those arising from the differentiation of mucoid and smooth forms. From the studies of Nutt (8) and later of Bisset (9) it has been apparent that an important distinction between the smooth and rough variants of a variety of bacterial species is their mode of cellular separation after division. The cells of smooth variants separate after division whereas those of rough variants do not, giving rise to filaments of great length. The chemical basis for this difference in behavior of smooth and of rough forms is not known at present but like surface polysaccharide, it, too, is apparently subject to quantitative variation. Such quantitative variation accounts, probably, for the colonial forms intermediate between smooth and rough described in a number of bacterial species, *i.e.* the so called rS, RS, and sR forms. It is evident from an examination of such variants that roughness and smoothness are not absolute cellular attributes but represent the more extreme variations in the pattern of cellular separation after division.

On the basis of the considerations just presented, it is apparent that significant difficulties may arise at times from attempts to classify bacterial variants on the basis of the appearance of colonies composed of each of the several forms. These difficulties may be obviated in some measure by considering certain aspects of bacterial variation in terms of cellular rather than of colonial morphology. By this procedure it is possible to define more exactly the nature of the bacterial variant studied and to eliminate the ambiguities which have arisen from attempts to fit different cellular types into a more or less rigid system of classification based upon colonial appearance. In the present report, some aspects of morphologic variation in pneumococcus are discussed and a hitherto undefined cellular variant is described.

Materials and Methods

Nomenclature of the Morphologic Variants of Pneumococcus.—Shortly after Arkwright's initial description of smooth and rough bacterial forms (10), two morphologic variants of pneumococcus were given these designations by Griffith (11). The smooth (S) variant of Griffith is isolated commonly from man infected with pneumococcus, is fully capsulated, and is characterized by cells which separate after division. Colonies of fully capsulated cells of this variant are typically mucoid in appearance but those of cells producing minimal amounts of type-specific capsular carbohydrate may be difficult to distinguish from colonies of non-

capsulated cells (6). The rough (R) variant described by Griffith is obtained from the capsulated variant by the selective cultural technique of growing it in the presence of homologous anticapsular antibody. The variant so selected differs from the capsulated form only in its inability to form capsular polysaccharide, and its divisional pattern is the same as that of the parent S cell. Both variants give rise to hemispherical colonies with smooth surface and margins. That the two morphologic forms of pneumococcus designated smooth (S) and rough (R) by Griffith might correspond respectively to the mucoid (M) and smooth (S) variants of other bacterial species was recognized by Hadley[1] who predicted that an additional morphologic variant of pneumococcus analogous to the rough (R) forms of other bacteria would be found. This prediction was confirmed by Dawson (1) who described a second non-capsulated variant of pneumococcus obtained from the non-capsulated R variant of Griffith by a selective cultural technique. This second non-capsulated variant of pneumococcus differs from the non-capsulated variant of Griffith by virtue of the fact that its cells remain attached following division, forming filaments of great length instead of separating to yield single cells and diplococci. The property of filamentous growth manifested by the non-

TABLE I

Nomenclature of Pneumococcal Morphologic Variants

Griffith (11)	Dawson (1)	Taylor (2)	Austrian and MacLeod (12)	Proposed
Smooth (S)	Mucoid (M)	Smooth (S)	Encapsulated (S)	Non-filamentous capsulated (*fil*− *S*+)
Rough (R)	Smooth (S)	Rough (R)	Griffith rough (GR)	Non-filamentous non-capsulated (*fil*− *S*−)
—	—	—	—	Filamentous capsulated (*fil*+ *S*+)
—	Rough (R)	Extreme rough (ER)	Dawson rough (DR)	Filamentous non-capsulated (*fil*+ *S*−)

capsulated variant of Dawson is responsible for the irregular surface and margins of colonies composed of such forms and for the autoagglutinability of the variant in liquid media. After describing the filamentous non-capsulated variant of pneumococcus, Dawson suggested that the colonial nomenclature of pneumococcus be revised so that the capsulated variant of pneumococcus designated smooth (S) by Griffith be known as mucoid (M), the non-capsulated variant called rough (R) by Griffith be designated smooth (S), and the newly described non-capsulated variant be named rough (R). Although this revised terminology of Dawson brings the nomenclature of pneumococcal colonial variants into conformity with that of a variety of bacterial species, it has never been widely employed; for there has been concern on the part of those working with pneumococcus that confusion might be created thereby. To avoid ambiguity, several additional modifications of nomenclature, none wholly satisfactory, have been proposed (2, 12) and these are recorded together with the earlier terminologies in Table I.

From the introductory remarks and the brief historical review, it is evident that any attempt to classify rigidly morphologic variants of bacteria on the basis of colonial appearance is beset with inherent difficulties and may result in inconsistencies when knowledge of the cellular bases for morphologic

[1] Hadley, cited by Dawson (1).

difference is lacking. For these reasons, it is proposed that definition of morphologic variation be made at the cellular rather than at the colonial level in order that problems consequent to classification in terms of colonial morphology may be avoided.

In the data to be presented, four morphologic variants of pneumococcus are described: non-filamentous capsulated, non-filamentous non-capsulated, filamentous capsulated, and filamentous non-capsulated. Filamentous forms differ from non-filamentous forms by virtue of the fact that the cells of the former fail to separate after division giving rise to chains of great length whereas those of the latter become detached from one another giving rise to single cells, diplococci, and short chains. Capsulated variants differ from non-capsulated variants in the ability of the former to synthesize type-specific surface polysaccharide, an ability lacked by the latter. To designate the several forms, a system of symbols is proposed which may be expanded to include a variety of cellular characters. Because a non-filamentous capsulated strain is the usual point of departure for the study of pneumococcal variation, the parent strain of a family of variants will be designated by a Roman numeral denoting its capsular type on isolation followed by a capital letter or letters indicating the specific strain. Pneumococcal characters for which the cytologic bases are unknown will be indicated in italics by letters or abbreviations in lower case; characters the bases of which are appreciated will be denoted in italics by letters or abbreviations in upper case. To designate a transformed character, the symbol indicating the altered cellular property will be followed by "*tr*". Intermediate variants will be denoted by "*int*" following the character designation. The symbols employed to designate the pneumococcal characters discussed are:—

filamentous: *fil*+
non-filamentous: *fil*−
capsulated: *S*I (the Roman numeral indicates capsular type)
non-capsulated: *S*−
M protein: *Mp1* (the Arabic numeral indicates protein type)

The four morphologic variants of an arbitrarily chosen strain of pneumococcus type III, strain IIID, are represented by the following symbols:—

non-filamentous capsulated —IIID (*fil*− *S*III)
non-filamentous non-capsulated—IIID (*fil*− *S*−)
filamentous capsulated —IIID (*fil*+ *S*III)
filamentous non-capsulated —IIID (*fil*+ *S*−)

If a particular study concerns transformation of the non-capsulated non-filamentous variant of strain IIID to capsular type IV, the transformed strain is indicated in the following fashion:—

IIID (*fil*− *S*IV*tr*)

The inclusion of additional characters such as M protein in the strain formula is optional and depends upon whether or not such an inclusion is pertinent to the data discussed. For example, the formula of the non-filamentous capsulated variant of strain IIID may be written: IIID (*fil*− *Mp3 S*III), denoting thereby that strain IIID possess type 3 M protein. Other characters may be included or omitted in similar manner.

The use of the conventions set forth permits an unambiguous mode of describing pneumococcal strains in terms of specific cellular properties and may be applied to the variants of other bacterial species.

Strains of Pneumococcus.—ISVI (*fil*− *S*I): a non-filamentous capsulated strain of pneumococcus type I carried for many years in the laboratory.

ID (*fil*− *S*I): a non-filamentous capsulated strain of pneumococcus type I isolated from a patient ill with lobar pneumonia at The Johns Hopkins Hospital in 1951.

IID39S (*fil*− *S*II): a non-filamentous capsulated strain of pneumococcus type II carried for many years in the laboratory. The non-filamentous non-capsulated variant of the strain, IID39S (*fil*− *S*−) used in these studies was formerly designated strain R36NC.

IIIA66 (*fil*− *S*III): a non-filamentous capsulated strain of pneumococcus type III carried for many years in the laboratory.

IVS (*fil*− *S*IV), VB (*fil*− *S*V), VIH (*fil*− *S*VI), VIIF (*fil*− *S*VII), and VIIIB (*fil*− *S*VIII): non-filamentous capsulated strains of pneumococcal capsular types IV, V, VI, VII, and VIII respectively, isolated from patients with lobar pneumonia at The Johns Hopkins Hospital in 1950–51.

Isolation of Morphologic Variants of Pneumococcus.—In the present studies, the non-filamentous capsulated variant of pneumococcus (*fil*− *S*+) has been the point of departure for the isolation of other morphologic variants.

The hitherto undefined filamentous capsulated variant (*fil*+ *S*+) was obtained from the non-filamentous capsulated form (*fil*− *S*+) by the selective cultural technique of Dawson (1). Neopeptone–meat infusion agar plates containing 5 per cent defibrinated rabbit's blood were inoculated on the surface at 1.0 cm. intervals by means of a fine wire needle bearing cells from an isolated clone of the non-filamentous capsulated form. The plates were then incubated at 36°C. for 7 to 14 days. Inspection of the cultures between 5 and 9 days revealed frequently the presence of fan-like excrescences at the margins of some colonies which on subculture directly onto a second blood agar plate gave rise to colonies with irregular surface and borders. Examination of the cells comprising the marginal outgrowths and the secondary colonies revealed them to be joined in filaments of great length and to be fully capsulated. The filamentous capsulated variants isolated in this fashion were maintained by serial passage at weekly intervals of the marginal cells of colonies incubated 7 days, the inocula being transferred from one blood agar plate to another by means of a needle.

Non-filamentous non-capsulated variants (*fil*− *S*−) were obtained in the usual way by the repeated transfer of the parent non-filamentous capsulated form in neopeptone–meat infusion broth containing 10 to 30 per cent homologous anticapsular rabbit serum. After a variable number of such transfers, non-filamentous non-capsulated variants were selected from platings of such cultures on blood agar.

Filamentous non-capsulated variants (*fil*+ *S*−) were obtained from non-filamentous non-capsulated variants by a selective cultural technique identical with that employed for the isolation of the filamentous capsulated variant from the non-filamentous capsulated form. Because of the instability of both filamentous variants in liquid media, no attempt was made to isolate filamentous non-capsulated variants from filamentous capsulated forms by growth of the latter in the presence of homologous capsular antiserum.

Preparation of Anti-M Protein and Anti-C Carbohydrate Sera, M Protein and C Carbohydrate Extracts, and Techniques of Precipitin Tests.—The methods used were those employed by Austrian and MacLeod (12).

Preparation of Transforming Principles and Technique of Transformation Reactions.—The methods employed were those described by MacLeod and Krauss (6).

Tests of Phagocytic Activity by Human Polymorphonuclear Leukocytes.—0.2 cc. of freshly drawn heparinized blood from a normal human subject was mixed with 0.1 cc. of a 16 hour culture of the appropriate strain of pneumococcus and 0.1 cc. of a 1:5 dilution of homologous anticapsular rabbit serum or normal rabbit serum. In tests with non-capsulated variants

0.1 cc. of normal saline solution was employed in place of serum. The mixtures were incubated 30 minutes at 36°C. in glass tubes following which time smears were made and stained with Wright's stain. The stained smears were examined under the oil immersion lens of a microscope for the presence of pneumococci within polymorphonuclear leukocytes.

EXPERIMENTAL

Description of the Filamentous Capsulated Variant of Pneumococcus.—By the use of the selective cultural technique of Dawson, filamentous capsulated variants were obtained from eight non-filamentous capsulated strains of pneumococcus representing all capsular types from I to VIII inclusive. Typically filamentous forms were not derived from the one strain, ISVI (*fil* — *S*I). In Fig. 1 is shown a portion of a colony of strain VIH (*fil* — *S*VI) after 7 days' incubation on a blood agar plate at 36°C. The rugose marginal outgrowth is readily differentiated from the more evenly surfaced parent colony. Preparations of cells from the central portion of the parent colony and from the marginal outgrowth, stained by the Gram technique, are shown in Figs. 2 and 3. The contrast between the cellular patterns in these two areas is evident, that of the central portion of the colony being characterized by non-filamentous growth and that of the peripheral excrescence by typical filamentous development. Examination of the cells from the latter area by the quellung technique reveals them to be fully capsulated as shown in Fig. 4. Similar observations were made on strains of other capsular types and the capsular material of the filamentous capsulated strains appeared similar in all respects to that of the parent non-filamentous capsulated forms in quellung, precipitin, and agglutination tests.

Direct subculture of marginal excrescences of filamentous capsulated variants onto new blood agar plates resulted in the perpetuation of the filamentous growth of the cells. Considerable variation in the appearance of colonies of capsulated filamentous forms was noted, however, which depended seemingly upon the length of filaments formed and the amount of capsular polysaccharide produced. In Figs. 5 and 6 are shown colonies of the non-filamentous and filamentous capsulated variants of a strain of pneumococcus type II on the same blood agar plate. The difference in appearance of the two morphologic variants is pronounced. In Figs. 7 and 8 the non-filamentous and filamentous non-capsulated variants are depicted for comparison and in Figs. 9 and 10 are shown the cells of these two non-capsulated variants. The dominant effect of the mode of cellular separation after division upon colonial appearance even in the presence of average amounts of capsular polysaccharide is evident. In Figs. 11 and 12, colonies of the filamentous and non-filamentous capsulated variants of pneumococcus type III are portrayed. Here the effect of filamentous growth on colonial structure is masked by the large amount of capsular polysaccharide produced by the cells until autolysis begins at which

time it becomes recognizable at the periphery of the colony. Although the colonies of the two morphologic variants of capsular type III are similar prior to autolysis, the difference in cellular pattern is readily apparent when microscopic preparations of the variants are examined before autolysis is manifest (Figs. 13 and 14). These findings bear out the importance of studying morphology at the cellular level. In liquid media, the filamentous capsulated variants yield agglutinated growth which varies among different strains from a flocculent sediment with limpid supernate to more diffuse granular growth throughout the medium.

In the transition from the non-filamentous to filamentous capsulated variant, changes in cellular morphology similar to those attributed by Dawson to the filamentous non-capsulated variant of pneumococcus were noted. Alterations in the axial relationships of the cell of two kinds were observed resulting in the appearance of either elongated bacillary forms or of plate-like forms in which the transverse axis of the cell exceeded its longitudinal axis by a factor of two or three. Filaments of the latter cell type resembled annelids (Fig. 15) in appearance and isolated cells of this form seemed more rectangular than circular in outline in quellung preparations.

When transferred repeatedly on solid media, the filamentous capsulated variants maintained their filamentous form but like non-filamentous capsulated strains, they tended on repeated subculture to give rise to variants producing progressively less capsular polysaccharide analogous to the intermediate capsular variants of non-filamentous capsulated forms. Continued subculture over a period of several months, therefore, resulted in the emergence at times of filamentous non-capsulated variants. In liquid media, repeated daily transfer was marked by the transition of filamentous capsulated to non-filamentous capsulated variants in a manner entirely analogous to the reversion of filamentous non-capsulated to non-filamentous non-capsulated variants described by Dawson, the number of transfers requisite for recognition of the change ranging between 8 and 20 for the strains examined. These observations on the behavior of filamentous capsulated variants under different environmental conditions suggest strongly that the pattern of cellular separation after division and the production of capsular polysaccharide are independently heritable cellular properties.

Demonstration through Transformation Reactions of the Genetic Independence of the Factors Controlling Cellular Separation after Division and Production of Capsular Polysaccharide in the Filamentous Capsulated Variant of Pneumococcus.—Previous studies (2–4, 13) have shown that, in pneumococcus, both the production of capsular polysaccharide and the pattern of cellular separation after division are subject to control through transformation reactions and that these two cellular attributes in the non-filamentous capsulated variant are independently heritable. If the genetic determinants of these two charac-

ters are distinct in the filamentous capsulated variant, it should be possible to demonstrate their independence also through transformation reactions. Such a demonstration should be effected most clearly by growth of the non-filamentous non-capsulated variant (*fil*− *S*−) in the presence of the transforming principles of a filamentous capsulated strain (*fil*+ *S*+), a reaction system which should yield both filamentous non-capsulated (*fil*+ *S*−) and non-filamentous capsulated (*fil*− *S*+) variants if the two genetic factors are indeed independent.

0.1 cc. quantities of a 10^{-4} dilution of an 18 hour blood broth culture of the non-filamentous non-capsulated pneumococcus, IID39S (*fil*− *S*−), were inoculated into small tubes containing 2 cc. of charcoal-absorbed neopeptone broth plus 5 per cent human pleural fluid and 0.1 cc. of the transforming principles of the filamentous capsulated strain, ID (*fil*+ *S*I), prepared according to the method of MacLeod and Krauss (6). Control tubes lacking transforming principles were inoculated also. After 24 hours' incubation at 37°C. the supernatant fluid and sedimented growth in each tube were streaked separately on the surface of neopeptone agar plates containing 5 per cent defibrinated rabbit's blood. The plates were examined after incubation overnight under a colony microscope at a magnification of 30 diameters and appropriate clones were isolated. In this fashion, the transformed non-filamentous capsulated strain, IID39S (*fil*− *S*I*tr*), and the transformed filamentous non-capsulated strain, IID39S (*fil*+*tr* *S*−) were recovered. A similar experiment performed with the transforming principles of the filamentous capsulated strain, VB (*fil*+ *S*V) yielded the non-filamentous capsulated variant, IID39S (*fil*− *S*V*tr*), and the filamentous non-capsulated variant, IID39S (*fil*+ *tr* *S*−).

The results establish conclusively the independent heritability of the two cellular attributes.

Antigenic Structure of the Morphologic Variants of Pneumococcus.—The antigenic structure of the four morphologic variants of pneumococcus was investigated by means of quellung and precipitin reactions. The non-filamentous and filamentous capsulated strains, IID39S (*fil*− *S*VIII*tr*) and IID39S (*fil*+ *S*VIII*tr*), both gave positive quellung reactions and formed disc-like precipitates in the presence of type VIII anticapsular rabbit serum. The non-filamentous and filamentous non-capsulated variants, IID39S (*fil*− *S*−) and IID39S (*fil*+ *S*−), failed to give quellung reactions and were agglutinated by rabbit antisera prepared with non-capsulated variants of pneumococcus. Lancefield extracts were prepared from each of these four strains and precipitin tests for C polysaccharide and M protein were carried out according to methods described previously in detail (12). The results are summarized in Table II. All four morphologic variants possess the species-specific C carbohydrate and a type-specific M protein. The capsulated variants differ from non-capsulated variants, however, by virtue of their ability to produce type-specific capsular polysaccharide.

Phagocytosis of the Morphologic Variants of Pneumococcus by Human Polymorphonuclear Leukocytes.—In these experiments the same filamentous and non-filamentous non-capsulated variants of a strain of pneumococcus type II

before and after transformation to capsular type VIII have been employed. As shown in Table III, both the non-capsulated variants are phagocytized by

TABLE II

Antigenic Structure of Morphologic Variants of Pneumococcus

Morphologic variant	Antigen		
	C polysaccharide	M protein	SSS
Non-filamentous capsulated IID39S (*fil*− *S*VIII*tr*)	+	+	+
Non-filamentous non-capsulated IID39S (*fil*− *S*−)	+	+	−
Filamentous capsulated IID39S (*fil*+ *S*VIII*tr*)	+	+	+
Filamentous non-capsulated IID39S (*fil*+ *S*−)	+	+	−

TABLE III

Phagocytosis of Morphologic Variants of Pneumococcus by Human Polymorphonuclear Leukocytes

Morphologic variant	Per cent PMN containing pneumococci	Average No. of pneumococci per PMN containing pneumococci	Average No. of pneumococci per PMN
Non-filamentous capsulated (*fil*− *S*+)			
Capsular antiserum present	100	4.9	4.9
" " absent*	0	0	0
Filamentous capsulated (*fil*+ *S*+)			
Capsular antiserum present	10	57.0	5.7
" " absent*	0	0	0
Non-filamentous non-capsulated (*fil*− *S*−)			
Added antiserum absent	80	7.8	6.2
Filamentous non-capsulated (*fil*+ *S*−)			
Added antiserum absent	20	32.5	6.5

* Normal rabbit serum was substituted for capsular antiserum in these controls.

human polymorphonuclear leukocytes in the absence of added antibody. In contrast, neither capsulated variant, filamentous or non-filamentous, is phagocytized in a glass system in the absence of homologous capsular antibody. Of additional interest is the difference in distribution among the leukocytes of phagocytized filamentous and non-filamentous pneumococci. Although cul-

tures of filamentous and non-filamentous pneumococcal variants grown under identical conditions were employed, the number of bacterial particles per unit volume of culture of the filamentous variants was significantly less than that of non-filamentous forms because of the greater degree of cellular aggregation of the former. Because of this fact, opportunities for collision between leukocyte and bacteria were less when filamentous variants were employed than when non-filamentous cells were used. This disparity is reflected in the per-

TABLE IV

Virulence of Morphologic Variants of Pneumococcus for the White Mouse

Amount of culture injected	Morphologic variant	
	Non-filamentous capsulated IID39S (*fil*− SVIII*tr*)	Filamentous capsulated IID39S (*fil*+ SVIII*tr*)
cc.		
10^{-2}	5/5	5/5
10^{-4}	5/5	4/5
10^{-6}	5/5	4/5
10^{-8}	5/5	2/5
Viable units/cc. (undiluted culture)	4×10^{8}	4×10^{7}
Amount of culture injected	Non-filamentous non-capsulated IID39S (*fil*− *S*−)	Filamentous non-capsulated IID39S (*fil*+ *S*−)
cc.		
10^{0}	0/4	0/4
10^{-1}	0/4	0/4
10^{-2}	0/4	0/4
10^{-3}	0/4	0/4
Viable units/cc. (undiluted culture)	2×10^{8}	4×10^{6}

Denominator of fraction represents number of mice injected intraperitoneally, numerator the number succumbing to pneumococcal infection within 2 weeks.

centage of leukocytes containing phagocytized pneumococci, being approximately 15 per cent when the leukocytes were exposed to filamentous forms and 90 per cent when exposed to non-filamentous forms. Despite this difference, the average number of pneumococci per leukocyte, based upon the total number of leukocytes examined, was approximately the same when either filamentous or non-filamentous variants were phagocytized because a leukocyte in contact with a bacterial filament engulfed many more bacteria than a leukocyte in contact with non-filamentous forms. These characteristic patterns of phagocytosis of filamentous and non-filamentous variants were noted whether the variants were capsulated or not.

Virulence of Morphologic Variants of Pneumococcus for the White Mouse.—

Virulence of pneumococci for the white mouse is dependent upon the presence of capsulation as is shown by the data in Table IV. Both non-capsulated variants, filamentous and non-filamentous, failed to cause death when injected in large numbers intraperitoneally into white mice. One viable bacterial unit of either capsulated variant of pneumococcus type VIII, filamentous or non-filamentous, was sufficient to produce a lethal infection in most animals. The somewhat less regular results observed with inocula of filamentous capsulated variants are doubtless the result in part of the less regular distribution of units of this variant in the higher dilutions of the infecting culture. Strains of pneumococci recovered from mice succumbing to infection with the filamentous capsulated type VIII strain retained their filamentous form after a single passage in this species. The importance of capsulation to the virulence of pneumococcus for the white mouse irrespective of the pattern of cellular separation after division is clearly evident from the experimental data.

DISCUSSION

The experiments described add a fourth morphologic variant, the filamentous capsulated form, to the three variants of pneumococcus in this category defined previously. Although it is highly probable that the filamentous capsulated variant of pneumococcus has been noted before, it has been without full appreciation of its significance. Bullowa and Wilcox (14) described the isolation from human beings ill with lobar pneumonia of capsulated pneumococci growing in long chains and giving rise to colonies with irregular surface and margins. Later, Dawson, in a personal communication to Hadley (15), reported the reversion of filamentous pneumococci to capsulated forms and may have been dealing originally with an intermediate capsular variant of a filamentous strain which mutated later to form more capsular carbohydrate, giving rise to "mucoid"-appearing colonies. That the ability to produce type-specific surface polysaccharide should be associated with filamentous forms in pneumococcus is not surprising, for there have been clear indications from previous experiments that patterns of cellular separation after division and production of capsular polysaccharide are independent characters (2, 4). The independence of these characters is corroborated by the present experiments and in addition, it has been shown that the production of capsular polysaccharide bears no obligatory linkage to either pattern of cellular separation after division. These observations are in accord with ones regarding several other bacterial species in which the production of surface polysaccharides has been found associated with filamentous forms (16–18). Although the antigenic structure of the different variants isolated is not described, the isolation of filamentous variants of Group B beta hemolytic streptococci from non-filamentous forms by Pomales-Lebrón and Morales-Otero (19) presents striking analogy to the isolation of the filamentous capsulated variant of pneumococcus.

As pointed out in the introductory remarks and later in the experimental section, there are good reasons for abandoning classification of the morphologic variants of bacteria on the basis of colonial appearance. Quantitative variation in the several characters responsible for colonial form may make accurate recognition of cellular structure difficult or impossible from examination of the colony alone. That mucoid colonies are dependent upon the production of significant quantities of surface polysaccharide by the cell is well established, but colonies of cells producing small amounts of such surface polysaccharide may lack mucoid appearance and require techniques other than simple inspection to establish their true nature. In addition, formation of large amounts of surface carbohydrate by the cell may mask completely, at the colonial level, the pattern of cellular separation after division necessitating again examination of component cells for identification of the morphologic variant. At present, little is known regarding the cellular bases responsible for the differences between filamentous and non-filamentous variants of bacteria. Recent studies of several bacterial species (20, 21) have suggested that divalent cations, notably magnesium and manganese, may play a significant role in influencing the pattern of cellular separation after division. In the absence of these metallic ions, non-filamentous forms behave phenotypically as filamentous forms. Unpublished observations (22) on pneumococcus indicate that non-filamentous variants of this species, too, will assume the filamentous pattern of growth when cultivated in media from which divalent cations have been largely removed by a chelating agent or by passage through an ion exchange resin column. In addition, filamentous growth of non-filamentous pneumococci may be induced by cultivation of the latter in liquid or on solid media containing certain quaternary ammonium compounds (23). The nature of the cell bridges resulting from growth in the presence of choline, however, is probably different from that existing in the genotypically filamentous forms, for colonies of pneumococci on choline-containing solid media do not assume the rugose appearance of the filamentous genotype although the cells grow in filamentous fashion. Thus a multiplicity of factors may be concerned with the ultimate expression of the pattern of cellular separation in bacteria. It may be hypothesized that non-filamentous forms possess a metal-activated enzyme or enzyme activator responsible for cellular separation after division which is lacking or reduced in filamentous forms. There is no evidence, however, to make these possibilities more probable than those predicating the possession by the filamentous variant of an enzyme producing an intercellular cement substance or of an inhibitor of the enzyme effecting cellular separation not possessed by non-filamentous forms. Solution of this basic problem requires further investigation.

The importance of the production of type-specific capsular polysaccharide to the virulence of pneumococcus has been reemphasized in the experiments reported here. It has been shown also quite clearly that the manifestation of

virulence by capsulated forms is independent of the pattern of cellular separation after division, both filamentous and non-filamentous capsulated variants producing lethal infections in mice when injected in small numbers. The association of virulence with antigenic structure rather than with the pattern of cellular separation after division may serve as a basis to explain certain apparent discrepancies in the behavior of morphologic variants of other bacterial species. Inasmuch as there appears to be no obligatory association of certain surface antigens with the patterns of cellular separation after division, there is no reason to anticipate obligatory linkages between characters of these two classes despite the fact that certain relationships between them are not uncommonly observed. Such an approach to the relation of virulence to bacterial variation should lead to the elimination of such distortions of terminology as the classification of the rough colonial variant of *Bacillus anthracis* as the "S" variant of that species (5). This is but another of the clarifications that should result from a return of the consideration of morphologic variation in bacteria from the colonial to the cellular level.

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SUMMARY

The problem of morphologic variation in pneumococcus has been reviewed and the desirability of studying such variation through an examination of bacterial cells rather than of bacterial colonies has been pointed out. To further this objective, a new terminology to describe the morphologic variants of pneumococcus, potentially applicable to other bacterial species, has been proposed.

A hitherto undefined morphologic variant of pneumococcus, the filamentous capsulated (*fil*+ *S*+) variant, has been defined and its relationship to the three previously recognized non-filamentous capsulated (*fil*− *S*+), non-filamentous non-capsulated (*fil*− *S*−), and filamentous non-capsulated (*fil*+ *S*−) variants has been presented.

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EXPLANATION OF PLATES

PLATE 4

FIG. 1. Portion of a colony of a non-filamentous capsulated variant of pneumococcus type VI after 7 days' incubation at 36°C. on blood agar. The rugose marginal outgrowth of the filamentous capsulated form appears at the upper margin of the colony. × 21.

FIG. 2. Cells from the central portion of the colony in Fig. 1 showing single cells and diplococci. Gram stain. × 1250.

FIG. 3. Cells from the marginal outgrowth from the colony in Fig. 1 showing the filamentous pattern of growth. Gram stain. × 1250.

FIG. 4. Cells from the marginal outgrowth of the colony in Fig. 1. Quellung preparation showing the presence of capsulation. × 1250.

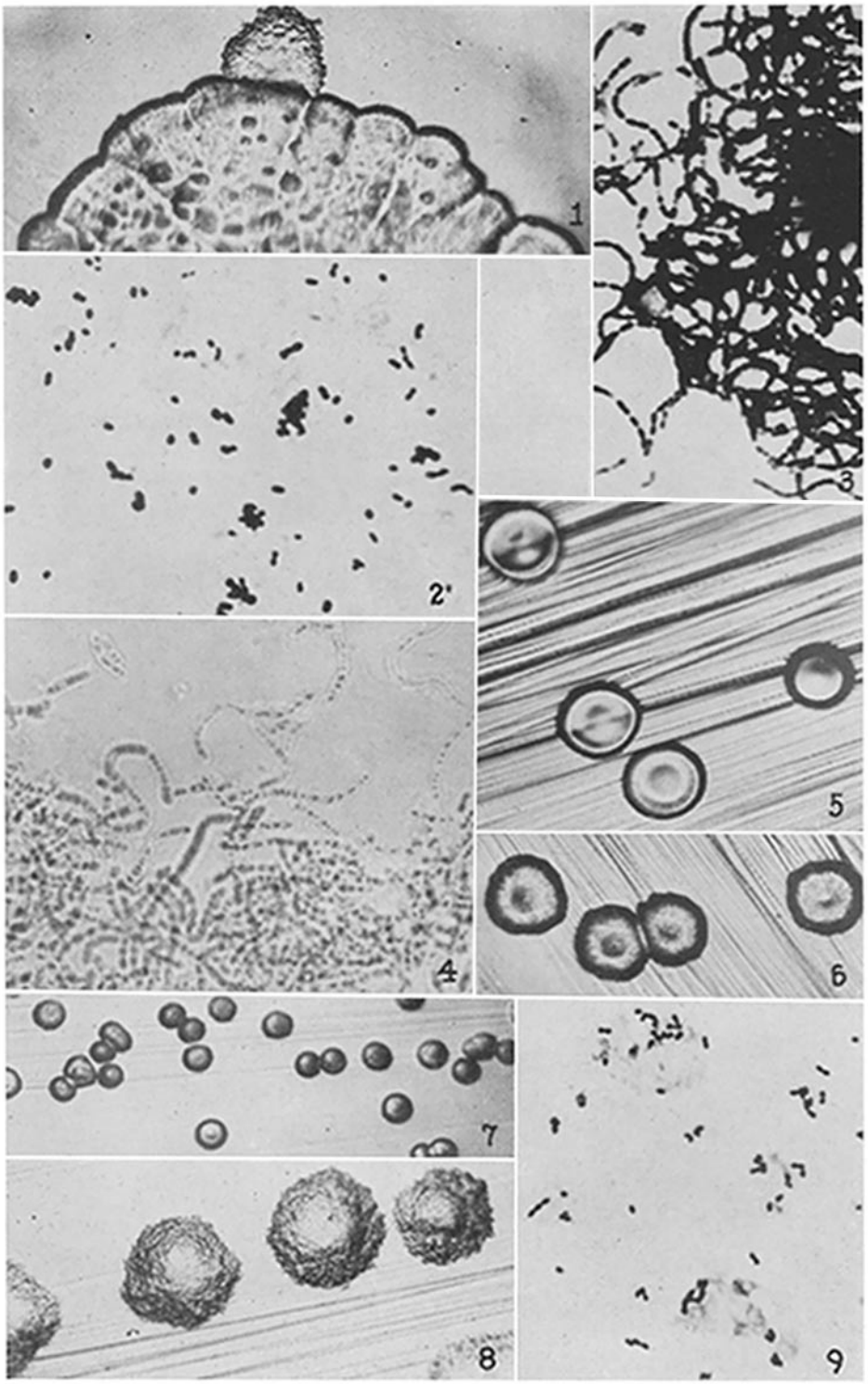
FIG. 5. Colonies of a non-filamentous capsulated variant of pneumococcus type II on blood agar after 24 hours' incubation at 36°C. × 18.

FIG. 6. Colonies of a filamentous capsulated variant of pneumococcus type II on blood agar after 24 hours' incubation at 36°C. × 18.

FIG. 7. Colonies of a non-filamentous non-capsulated variant of pneumococcus type II on blood agar after 24 hours' incubation at 36°C. × 18.

FIG. 8. Colonies of a filamentous non-capsulated variant of pneumococcus type II on blood agar after 24 hours' incubation at 36°C. × 18.

FIG. 9. Cells of the non-filamentous non-capsulated variant of pneumococcus type II. Gram stain. × 900.



(Austrian: Morphologic variation in pneumococcus. I)

PLATE 5

FIG. 10. Cells of the filamentous non-capsulated variant of pneumococcus type II. Gram stain. × 1100.

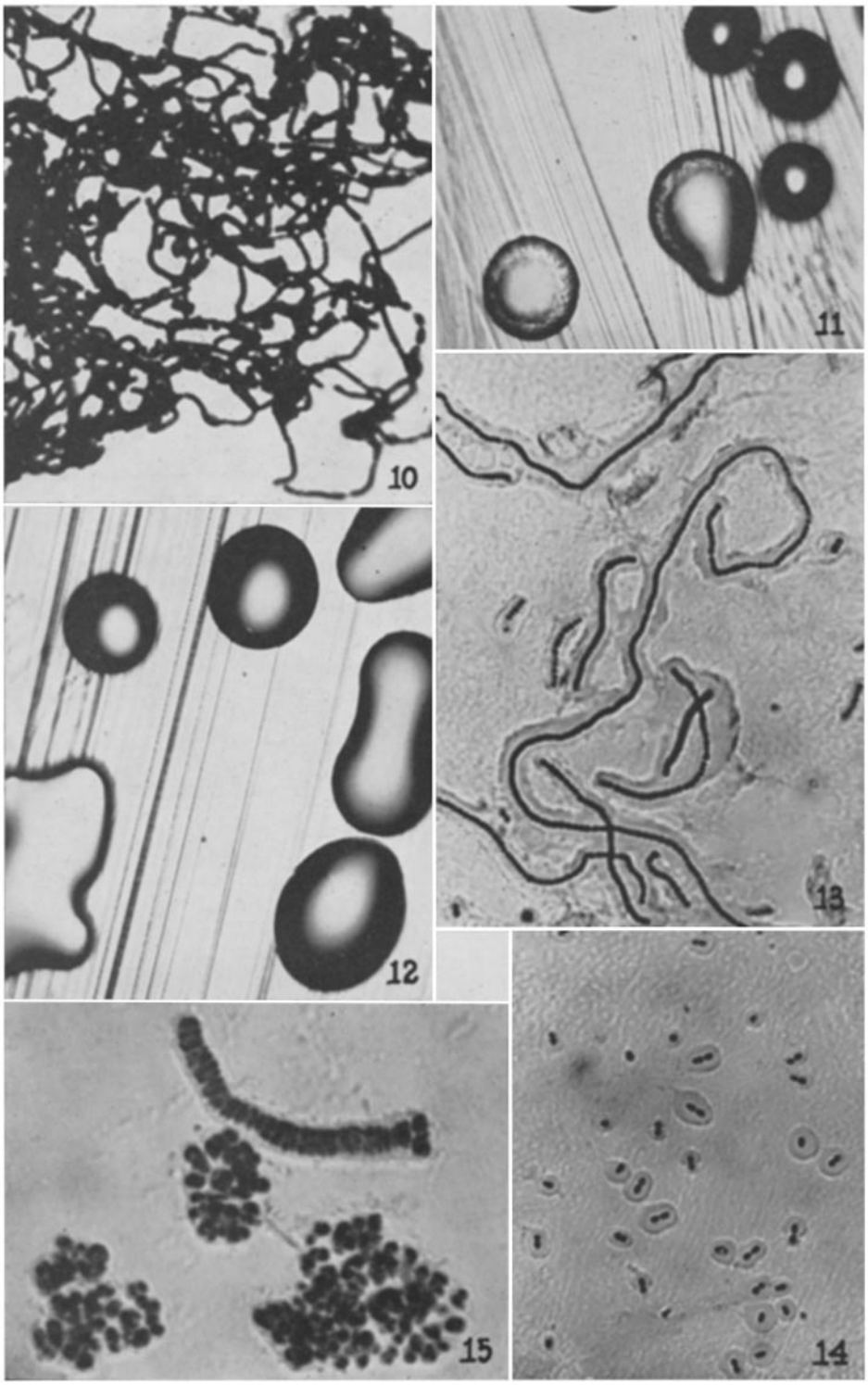
FIG. 11. Colonies of a filamentous capsulated variant of pneumococcus type III on blood agar after 24 hours' incubation at 36°C. Irregularity of colonial surface visible only in partially autolysed colonies. × 21.

FIG. 12. Colonies of a non-filamentous capsulated variant of pneumococcus type III on the same blood agar plate as colonies in Fig. 11. Autolysis fails to reveal surface irregularity. × 21.

FIG. 13. Quellung preparation of cells from a colony shown in Fig. 11 revealing filamentous forms. × 1100.

FIG. 14. Quellung preparation of cells from a colony depicted in Fig. 12 showing single cells and diplococcal forms. × 1100.

FIG. 15. Quellung preparation of a filamentous capsulated variant of pneumococcus type I showing a chain of plate-like cells resembling an annelid. × 1980.



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